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EPR demonstration of iron–nitrosyl complex formation by cytotoxic activated macrophages

(nitric oxide/iron-sulfur proteins/activated macrophage cytotoxicity)

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ABSTRACT **Activated macrophage cytotoxicity is characterized by loss of intracellular iron and inhibition of certain enzymes that have catalytically active nonheme-iron coordinated to sulfur. This phenomenon involves the oxidation of one of the terminal guanidino nitrogen atoms of L-arginine, which results in the production of citrulline and inorganic nitrogen oxides (NO_2^-, NO_3^-, and NO). We report here the results of an electron paramagnetic resonance spectroscopic study performed on cytotoxic activated macrophage (CAM) effector cells, which develop the same pattern of metabolic inhibition as their targets. Examination of activated macrophages from mice infected with *Mycobacterium bovis* (strain bacillus Calmette–Guérin) that were cultured in medium with lipopolysaccharide and L-arginine showed the presence of an axial signal at g = 2.039, which is similar to previously described iron–nitrosyl complexes formed from the destruction of iron-sulfur centers by nitric oxide (NO). Inhibition of the L-arginine-dependent pathway by addition of N^G-monomethyl-L-arginine (methyl group on a terminal guanidino nitrogen) inhibits the production of nitrite, nitrate, citrulline, and the g = 2.039 signal. Comparison of the hyperfine structure of the signal from cells treated with L-arginine with terminal guanidino nitrogen atoms of natural abundance N^{14} atoms or labeled with N^{15} atoms showed that the nitrosyl group in this paramagnetic species arises from one of these two atoms. These results show that loss of iron-containing enzyme function in CAM is a result of the formation of iron–nitrosyl complexes induced by the synthesis of nitric oxide from the oxidation of a terminal guanidino nitrogen atom of L-arginine.**

Recent evidence implicates the biologic synthesis of nitric oxide in certain murine macrophage effector cell functions. Macrophages activated *in vivo* (most commonly by infection of the host with the intracellular pathogen *Mycobacterium bovis* strain bacillus Calmette–Guérin) are cytotoxic to tumorigenic cells *in vitro* by an antibody-independent nonphagocytic mechanism after exposure to a second signal such as certain bacterial products (e.g., lipopolysaccharide) and macrophages that are not activated are not cytotoxic (1, 2). This effect is probably an important factor in host immunity to tumors (3). It has been shown (4–7) that an important event in this phenomenon is the induction of loss of target-cell intracellular iron, which results in the elimination of many iron-containing enzyme functions, including mitochondrial electron transfer [NADH:ubiquinone oxidoreductase and succinate:ubiquinone reductase (5–9)], aconitase (5, 6), and DNA synthesis (10, 11). A common feature of these enzymes is the existence of catalytically active nonheme iron (5–7, 12). These cytotoxic activated macrophage (CAM)-induced biochemical changes require the presence of L-arginine and are inhibited by N^G-monomethyl-L-arginine (methyl group on a terminal guanidino nitrogen) (6, 13–15). These cytotoxic functions are mediated by a pathway synthesizing nitrite/nitrate (NO_2^-/NO_3^-) from a terminal guanidino nitrogen atom of L-arginine, which is converted to L-citrulline without loss of the guanidino carbon (6, 14, 16–19). CAM also can synthesize nitric oxide (NO) (18, 19, 20), a paramagnetic stable free radical, and reagent NO induces the same pattern of metabolic inhibition as CAM (18, 21). Earlier work has demonstrated that iron-sulfur groups are sensitive targets for iron–nitrosyl complex formation (22–26). In addition, CAMs develop the same pattern of enzymatic inhibition as their target cells (6). This suggests that iron–nitrosyl complex formation could occur in the CAM effector cells as well as in their target cells. Utilizing electron paramagnetic resonance (EPR) spectroscopy, we describe here iron–nitrosyl complex formation in CAM. These findings provide a molecular explanation for iron loss and enzyme inhibition induced by NO synthesized by CAM from L-arginine.

MATERIALS AND METHODS

Culture Medium. A modification of Dulbecco's modified Eagle's medium (DMEM) was made for the experiments reported here. Basic DMEM contained the following components: DMEM salts (same concentration as commercial DMEM), 100 units of penicillin per ml, 100 μg of streptomycin per ml, 1:50 dilution of concentrated modified Eagle's medium vitamin solution, 2.5 g of $NaCO_3$ per liter, 20 mM glucose, 4 mM L-glutamine, and 20 ng of lipopolysaccharide per ml. Other components added to basic DMEM are described with each experiment and included: 1.5 mM L-[*guanidino*-$^{14}N_2$]arginine, 1.5 mM L-[*guanidino*-$^{15}N_2$]-arginine (Cambridge Isotope Laboratories, Woburn, MA), 0.5 mM N^G-monomethyl-L-arginine (acetate salt) (Chem-Biochem Research, Inc., Salt Lake City, UT), and 0.5 mM L-cystine.

Preparation of CAM. To obtain activated macrophages, 6- to 12-week-old C_3H/HeN female mice were infected by the i.p. route with 5×10^6 colony-forming units of the Pasteur strain of bacillus Calmette–Guérin (BCG) suspended in 1 ml of 0.9% saline 28 days before removing the peritoneal exudate cells (PEC) (27). Four days before PEC removal, the mice received a second i.p. inoculation of 5×10^6 colony-forming units of BCG suspended in 1 ml of 10% (vol/vol) peptone broth. Treatment of these *in vivo* activated macrophages with small amounts of lipopolysaccharide (20 ng/ml or less) *in vitro* induces them to become cytotoxic for tumor cells (27–31), and we define them as CAM. Stimulated macrophages were obtained from normal mice that were inoculated with 1 ml of thioglycolate broth (Difco) 4 days before harvest. Stimulated macrophages, like normal perito-

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Abbreviations: CAM, cytotoxic activated macrophage; PEC, peritoneal exudate cells; BCG, bacillus Calmette–Guérin.

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neal macrophages, are not cytotoxic for tumor cells and do not synthesize inorganic nitrogen oxides from L-arginine (14).

Preparation of Macrophage Cultures for Measurement of Iron–Nitrosyl Complexes by EPR. Macrophage monolayers were prepared by adherence of PEC to Costar 3100 tissue-culture dishes (9×10^7 PEC per dish from BCG-infected mice and 4.5×10^7 PEC per dish from thioglycolate-elicited normal mice) in 25 ml of basic medium, without other additions, for 1 hr. The PEC from BCG-infected mice were 50–60% macrophages, and the PEC from normal mice inoculated with thioglycolate broth were >90% macrophages. Nonadherent PEC were removed by washing twice with basic DMEM. Each experimental group, consisting of five Costar 3100 tissue culture dishes containing macrophage monolayers, was supplemented with appropriate additions and cultured at 37°C in 5% CO_2/95% air for 20.5 hr. At this time, the culture supernatant was decanted, the macrophages were removed from the tissue culture substrate with a rubber policeman, and a thick cell suspension was added to quartz EPR tubes. Similar volumes of medium were used to suspend the macrophages to ensure equal cells per ml in the EPR tubes.

Measurement of Inorganic Nitrogen Oxides and L-Citrulline Synthesized by CAM. Nitrate in the culture supernatant was reduced to nitrite by using nitrate reductase prepared from *Escherichia coli* (ATCC 25922) by the method of Bartholomew (32). The total nitrate plus nitrite in the culture supernatant, after conversion of nitrate to nitrite with the reductase, was measured with the Greiss reagent [final concentrations in the reaction mixture: 0.33% sulfanilamide/0.03% *N*-(1-naphthyl)ethylenediamine hydrochloride/30% acetic acid]. Nitrite forms a chromophore with the Greiss reagent absorbing at 543 nm and is quantitated spectrophotometrically. Nitrite was also measured independently of nitrate in samples of culture supernatant not incubated with nitrate reductase. L-Citrulline was measured by a colorimetric reaction that detects carbamido compounds (33). Urea was removed from samples before this assay by incubation with urease (1 unit/ml) at 27°C for 1 hr; the assay was performed as described (14).

EPR Spectroscopy. EPR spectra were recorded at 77 K on a Varian E-109 spectrometer equipped with a 9.5-GHz microwave bridge. The field was calibrated periodically by utilizing a sample of reduced methyl viologen. The power was 1 mW, and modulation frequency was 100 kHz. The microwave frequency was 9.15 GHz. Instrument gain and magnetic-field-modulation amplitude are specified in the figure legends.

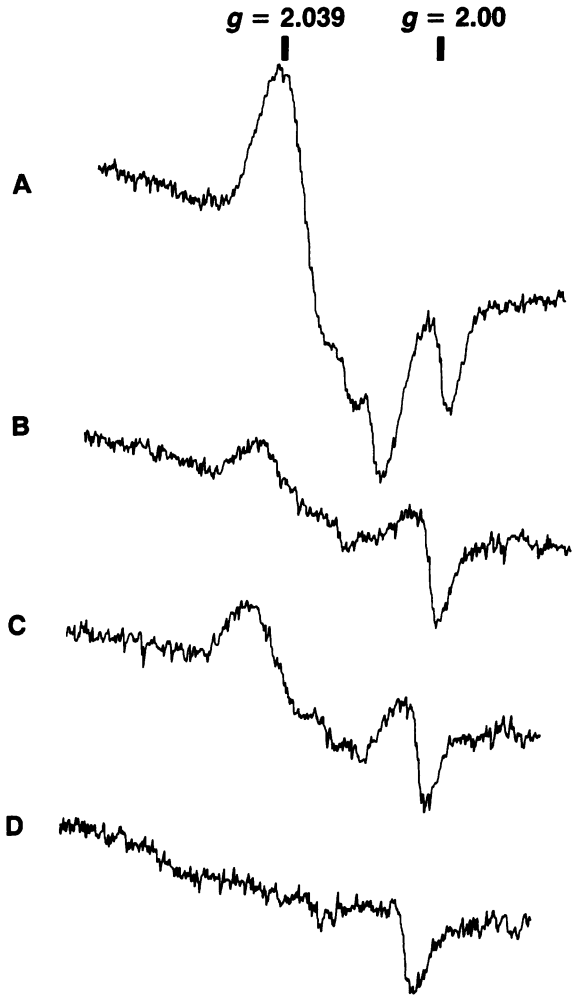
RESULTS

When NO (a paramagnetic molecule) complexes with iron, the unpaired electron interacts with the *d* orbitals of the metal atom, and the EPR spectrum is characteristically shifted away from the free electron value of $g = 2.0023$ as a result of orientation-dependent contributions to the effective magnetic moment of the electron. Since paramagnetic molecules are rare in biological systems, EPR thus can be used to detect the presence of iron–nitrosyl (Fe·NO) complexes in cells. Indeed, this technique has been used previously to demonstrate the destruction of iron–sulfur centers in bacteria upon the addition of nitrite (which is reduced to NO) with the simultaneous formation of a feature at $g = 2.035$ ascribable to the presence of Fe·NO complexes (26).

In the experiments reported here, CAMs were used, which have been shown to develop the same pattern of metabolic inhibition (i.e., L-arginine-dependent loss of iron-containing enzyme function) as their targets (6). Fig. 1*A* shows the EPR spectrum at 77 K of whole CAM after activation in the presence of 1.5 mM L-arginine and 20 ng of LPS per ml. The axial feature at $g = 2.039$ is virtually identical to previously published spectra for iron–nitrosyl complexes of a variety of iron-containing proteins, peptides, and amino acids (22, 23, 26, 34–36). Although nitric oxide forms an EPR-visible complex with heme [e.g., hemoglobin (37) and cytochrome a_3 (38)], these species typically exhibited a different symmetry (rhombic vs. axial), a wider range of *g* values, and a usually resolved nitrogen hyperfine triplet structure, and their EPR spectra typically extend upfield to significantly less than $g = 2$. Also shown in this figure are the results of analyses that showed the concomitant synthesis of nitrite, nitrate, and citrulline. The simultaneous decrease in the signal intensity as well as the amount of nitrite, nitrate, and citrulline produced when N^G-monomethyl-L-arginine was added (Fig. 1*B*) shows that the presence of this paramagnetic species is a result of the L-arginine-dependent pathway. Fig. 1*C* (no added L-arginine) shows that there was endogenous L-arginine present intracellularly; again, the amplitude of the signal reflected the activity of the L-arginine-dependent pathway, as determined by the production of nitrite, nitrate, and citrulline. Finally, Fig. 1*D* shows that virtually complete elimination of nitrite, nitrate, and citrulline production from endogenous L-arginine by N^G-monomethyl-L-arginine resulted in the prevention of the appearance of the signal. Although not shown, stimulated macrophages (i.e., not exposed to cytokines or lipopolysaccharide) did not show this signal even in the presence of L-arginine. Although an isotropic signal also appeared at $g = 2.00$, this radical species was not associated with the macrophage cytotoxic response, as judged by the lack of effect of the treatments above on the signal amplitude. These results show the formation of an EPR-detectable signal ascribable to iron–nitrosyl complex formation that is dependent on the metabolism of L-arginine to citrulline and inorganic nitrogen oxides.

To establish definitively the origin of the nitrosyl group in this EPR-visible species, we treated CAM with L-arginine containing terminal guanidino nitrogen-14 or nitrogen-15 atoms and examined the signal at lower magnetic field modulation (3.2 vs. 10 G) to detect small differences in hyperfine structure. This difference in instrument settings is the reason why the signal in Fig. 2*A* is smaller in amplitude than in Fig. 1*A*. As shown by comparison in Fig. 2 of *B* with *A*, addition of L-cystine to the CAM resulted in a significant increase in the amount of paramagnetic signal produced in these cells as well as the appearance of an additional feature at a *g* value slightly above 2.00. These increases in signal were reflected by an increase in the synthesis of nitrite, nitrate, and citrulline upon L-cystine addition (in separate, unpublished studies it was observed that L-cystine and L-cysteine enhance inorganic nitrogen oxide and L-citrulline synthesis from L-arginine by CAM; J.B.H., R. Taintor, and Z. Vavrin). This result raises the possibility that at least part of the signal may be due to the presence of a small molecular weight complex of iron, nitric oxide, and thiolate anion-containing ligand(s). The EPR properties of such small molecular weight complexes have been described previously, and the spectra resemble those presented here (34–36, 39–46), although the molecular nature of the species giving rise to these signals has not been delineated.

In addition to the applied magnetic field, an unpaired electron will be influenced by the presence of any neighboring magnet, including nuclei. This means that changes in the EPR spectrum upon changing the magnetic properties of a nucleus (isotopic substitution) can be used to determine the identity of the nuclei in close proximity to the unpaired electron. Comparison of the features of the $g = 2.039$ species obtained with terminal guanidino nitrogen atoms of L-arginine of natural abundance ^{14}N ($I = 1$, Fig. 2*B*) with those labeled with ^{15}N ($I = 1/2$, Fig. 2*C*) reveals differences in hyperfine structure. Because this signal is quite possibly a result of more than one iron–nitrosyl species present in these cells, specific assignment of the nitrogen hyperfine interac-



Treatment	NO_2^-, nmol/10^7 cells	NO_3^-, nmol/10^7 cells	Citrulline, nmol/10^7 cells
+L-Arg	425.5	569.4	650.0
+L-Arg + N^GMMA	66.7	68.1	142.2
No addition	108.3	81.5	221.3
+ N^GMMA	8.8	24.5	20.4

FIG. 1. L-Arginine-dependent simultaneous formation of nitrite, nitrate, citrulline, and the g = 2.039 signal by cytotoxic activated macrophages. After a 20.5-hr incubation with the indicated additions, cells were treated and analyzed for nitrite, nitrate, and citrulline as described. Whole-cell aliquots were examined by EPR spectroscopy as described with a modulation amplitude of 10 G (1 G = 0.1 mT) and a relative instrument gain of 1.6×10^3. N^GMMA, N^G-monomethyl-L-arginine.

tions displayed in this figure is not straightforward. In particular, the largely unresolved hyperfine structure in this spectrum may be due to additional splitting from methylene protons adjacent to thiolate sulfur(s) in a small molecular weight thiolate anion–iron–nitrosyl complex, as has been demonstrated previously (34). Regardless of the exact nature of the hyperfine interactions involved, however, this result shows conclusively that the nitrosyl moiety in this paramagnetic complex (or complexes) originates from a terminal guanidino nitrogen atom of L-arginine.

DISCUSSION

In addition to a role in macrophage effector cell functions, NO mediates several bioregulatory activities. For example, before the biologic synthesis of NO was discovered, it was known that organic nitrates and authentic NO can increase cGMP levels by forming a nitrosyl heme moiety that associates with and activates soluble guanylate cyclase (47, 48). Recently it has been found that NO synthesized from L-arginine by an enzymatic pathway similar to that existing in macrophages also functions as a signal, causing activation of soluble guanylate cyclase. Endothelial cell-dependent vascular smooth muscle relaxation and inhibition of platelet aggregation (in the presence of cyclooxygenase inhibition) (49–51) as well as *N*-methyl-D-aspartate-induced elevation of cyclic GMP in neural tissue (52, 53) are examples of modulation of cellular function via signal transduction by NO synthesized from L-arginine by a constitutive pathway.

The pathway synthesizing NO from L-arginine that we examined in this study is an effector limb of the cell-mediated immune response in murine cells (13, 14, 18, 21). However, this pathway is not constitutive but is induced by cytokines in macrophages and other cells not specialized for host defense (13, 14, 17, 54). A further difference between this and the constitutive pathway is the much larger amount of nitric oxide produced that, as the results here show, form nonheme iron–nitrosyl complexes that are readily detected by EPR spectroscopy. This pathway causes mobilization of intracellular iron (4–7) and inhibition of enzymes with catalytically active iron–sulfur groups (5–9). Recent experiments have shown that NO is the precursor of NO_2^-/NO_3^- synthesized and the likely effector molecule causing the biochemical changes induced by CAM (18–20). The appearance of a characteristic EPR signal in CAM reported here and the inhibition of the appearance of this signal when the L-arginine-dependent pathway is inhibited by N^G-monomethyl-L-arginine show that the biochemical pattern of inhibition induced in CAM can be explained by the destruction of iron-sulfur centers (with consequent loss of enzymatic activity) resulting from the formation of iron–nitrosyl complexes. The identification of iron–nitrosyl complexes in this study links iron, which is known to be lost from cells that develop activated macrophage-induced cytotoxicity (4–7), and NO,

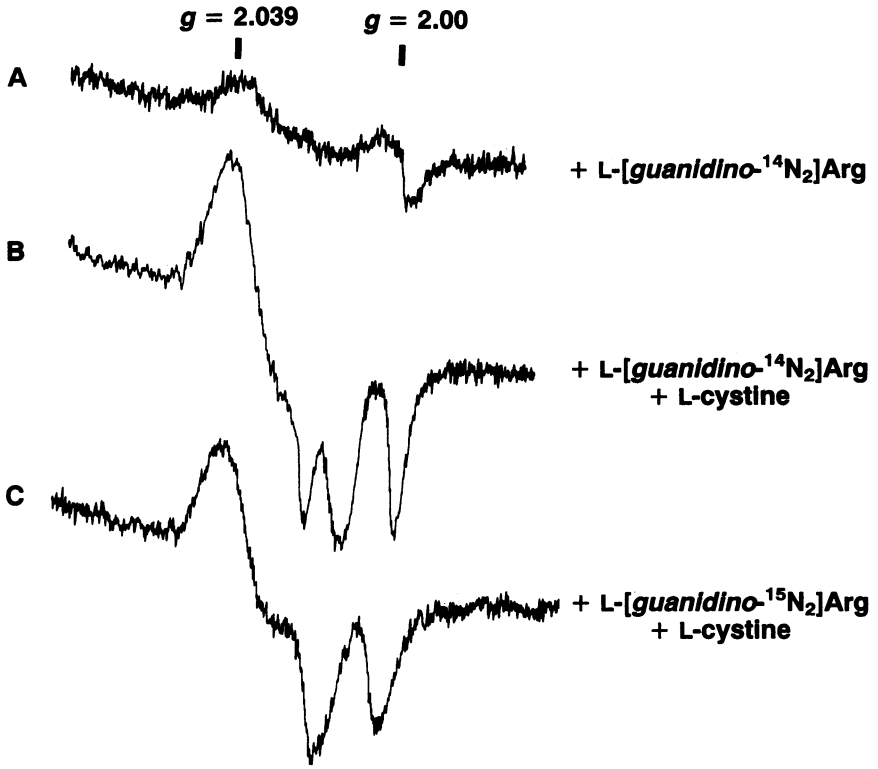


FIG. 2. Effects of L-cystine and L-arginine guanidino nitrogen isotopic substitution on the $g = 2.039$ signal in cytotoxic activated macrophages. Cells were treated as described for Fig. 1 and in *Materials and Methods*. Modulation amplitude was 3.2 G, and relative instrument gain was 3.2×10^3.

which is synthesized by activated macrophages capable of expressing cytotoxicity (18–20).

These findings show that NO synthesized from L-arginine functions in two ways. It is a cytotoxic effector molecule that causes enzymatic inhibition via iron–nitrosyl complex formation and, in addition, an intracellular messenger that activates soluble guanylate cyclase. The quantity of NO synthesized as well as other factors may determine the type of biological activity observed.

Formation of EPR-detectable iron–nitrosyl complexes of the general formula $[Fe(NO)_2SR_2]^-$ from iron-sulfur proteins under mild conditions have been reported; these species are formed by addition of nitrite or nitric oxide (22, 23, 26, 55) and also have been reported to be associated with tumor formation in animals induced by the administration of chemical carcinogens (55–61) as well as by tumor transplantation (L. H. Piette, personal communication). The signal was induced by a variety of chemically different carcinogens, and its appearance was enhanced by the addition of nitrite to the diet. The results presented here suggest that at least part of this signal may be attributable to an immune response to neoplastic transformation. A similar signal has also been reported in plant material upon prolonged storage (62, 63), and there appeared to be a correlation between the appearance of this species and the incidence of esophageal cancer. In the case of nitrite treatment of *Clostridium botulinum* (as a model for the prevention of botulism by the addition of nitrites to meals), it has been shown that disappearance of the $g = 1.94$ EPR signal from iron-sulfur proteins occurs concomitant with the appearance of the iron–nitrosyl signal (26), implying stoichiometric conversion. Enzymatic studies documenting the loss of iron-sulfur enzymatic function have supported this possibility further (64) and suggest close analogy to the CAM system reported here. The EPR properties of small molecular weight inorganic complexes containing iron–nitrosyl groups have been described (34–36, 39–46), and these species exhibit spectra that are quite similar to those reported here. At the present time, it is unknown whether the species that gives rise to the spectra reported here is protein-bound or a smaller complex, perhaps similar in structure to Roussin's salts. The increase in signal production upon the addition of L-cystine could suggest the involvement of thiolate anion as ligand and explain the CAM-induced loss of intracellular iron.

Note Added in Proof. Findings similar to those reported here have been obtained by C. Pellat *et al.* (65).

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